

# Cellular Redistribution of Inducible Hsp70 Protein in the Human and Rabbit Heart in Response to the Stress of Chronic Hypoxia

ROLE OF PROTEIN KINASES\*

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Many infants who undergo cardiac surgery have a congenital cyanotic defect where the heart is chronically perfused with hypoxic blood. Infant hearts adapt to chronic hypoxemia by activation of intracellular protein kinase signal transduction pathways. However, the involvement of heat shock protein 70 in adaptation to chronic hypoxemia and its role in protein kinase signaling pathways is unknown. We determined expression of message and subcellular protein distribution for inducible (Hsp70i) and constitutive heat shock protein 70 (Hsc70) in chronically hypoxic and normoxic infant human and rabbit hearts and their relationship to protein kinases. In chronically hypoxic human and rabbit hearts message levels for Hsp70i were elevated 4- to 5-fold compared with normoxic hearts. Hsp70i protein was redistributed from the particulate to the cytosolic fraction. In normoxic infants Hsp70i protein was distributed almost equally between the cytosolic and particulate fractions. Hsc70 message and subcellular distribution of Hsc70 protein were unaffected by chronic hypoxia. We then determined if protein kinases influence Hsp70i protein subcellular distribution. In rabbit hearts SB203580 and chelerythrine reduced Hsp70i message levels, whereas SB203580, chelerythrine, and curcumin reversed the subcellular redistribution of Hsp70i protein caused by chronic hypoxia, with no effect in normoxic hearts, indicating regulation of Hsp70i message and subcellular distribution of Hsp70i protein in chronically hypoxic rabbit hearts is influenced by protein kinase C and mitogen-activated protein kinases, specifically p38 MAPK and JNK. We conclude the Hsp70 signal transduction pathway plays an important role in adaptation of infant human and rabbit hearts to chronic hypoxemia.

Each year, more than 25,000 children undergo corrective surgery for cardiac birth defects. Advances in surgical techniques have made primary correction or palliation of almost all congenital cardiac defects possible. Early surgical intervention is important to promote more normal development. Many children undergoing cardiac surgery in the first year of life have cyanotic heart defects where the myocardium is chronically perfused with hypoxic blood. By elucidating the impact that prolonged periods of hypoxemia exert upon resistance to subsequent ischemia, we should be able to understand and improve cardioprotection in children with congenital heart defects. Recently we showed that infant human and rabbit hearts adapt to chronic hypoxemia by activation of PKC, p38 MAPK, and JNK signaling pathways (1). As chronic hypoxemia in rabbits induces changes in the heart similar to that found in humans, the rabbit may be useful to test other adaptive mechanisms thought to occur in human.

Heat-shock proteins are self-protective proteins that maintain cell homeostasis against various forms of stress as an adaptive response (2). These proteins are induced by a wide variety of stressors and have broad cytoprotective functions. The 70-kDa family of heat shock proteins (Hsp70), in particular, plays a vital role in cellular protection and has been detected in various tissues subjected to stress (3, 4). The Hsp70 stress proteins exist as two isoforms in eukaryotic and prokaryotic cells. Messenger RNA and protein for the cognate form of Hsc70 is constitutively expressed in the nonstressed cell. The Hsc70 isoform is slightly inducible and functions as a molecular chaperone. However, a slightly different form of Hsp70, Hsp70i, is highly inducible and up-regulates in response to stressful stimuli to function as a molecular chaperone. Hsp70 protein in unstressed cells is found in the cytoplasm and nucleus (5) but redistributes to the cytoplasm in response to stress. Overexpression of mRNA for Hsp70 using gene therapy translates to increased Hsp70 protein levels that are associated with increased cardioprotection, suggesting this stress protein plays an important role in mediating resistance to myocardial ischemia (6). Adaptation to the stress of chronic hypoxia also

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† The abbreviations used are: PKC, calcium-dependent protein kinase; PKA, cyclic AMP-dependent protein kinase; PKG, cyclic GMP-dependent protein kinase; Hsp70i, inducible heat shock protein 70; Hsc70, constitutive heat shock protein 70; HSF1, heat shock transcription factor; HSE, heat shock element; Hsp27, heat shock protein 27; Hsp32, heat shock protein 32; Hsp72, heat shock protein 72; MAPK, mitogen-activated protein kinase; JNK, c-Jun NH<sub>2</sub>-terminal kinase; TBS, Tris-buffered saline; TBSS, TBS with saponin; RT, reverse transcriptase.

results in increased cardioprotection (1, 8). We therefore reasoned that adaptation to chronic hypoxia would result in increased expression of mRNA and subcellular redistribution of Hsp70 protein.

Hsp70 expression is controlled in part by protein kinases. Activation of PKC, p38 MAPK, and JNK signaling pathways appears to confer cardioprotection in infant rabbit hearts adapted to chronic hypoxia (1). However, the relationship of Hsp70 to these protein kinase signaling pathways following adaptation to chronic hypoxemia is unknown. Ohnishi et al. (7) examined the involvement of protein kinases in the regulation of Hsp70i gene expression in the A-172 cell line with use of H-7, a potent inhibitor of PKC, PKA, and PKG, and have shown decreased Hsp70i expression. Treatment of rat hearts with chelerythrine, a PKC inhibitor, prior to heat shock increases infarct size compared with non-heat shock-treated animals (8). In addition, there was a marked increase in Hsp70i expression in the heat shock-treated group. Pretreatment with chelerythrine failed to inhibit the expression of Hsp70i, suggesting that heat shock-induced ischemic tolerance is mediated via the PKC pathway, and this protection does not appear to be directly related to the expression of Hsp70i in rat heart (8). The MAPK family plays an important role in coordinating gene responses to various stresses. Induction of Hsp70 in the 9L rat is preceded by phosphorylation and activation of p38 MAPK and p42/44 MAPK. Specific inhibitors of p38 MAPK (SB203580) and p42/44 MAPK (PD98059) are known to eliminate Hsp70 induction (9).

Phosphorylation and activation of Hsp27, a substrate for p38 MAPK, is present in chronically hypoxic infant hearts but not in normoxic hearts (1). Overexpression of Hsp27 in myocytes confers protection against simulated ischemia (10). Another small molecular weight stress protein, Hsp32, has been shown to play an important role in cardiovascular adaptation to acute hypoxic stress (11). We reasoned that Hsp32 might also play a role in adaptation of infant human and rabbit heart to the stress of chronic hypoxia.

Chronic hypoxia represents a significant stress to the heart. However, the role of heat shock proteins in mediating adaptation of the heart to chronic hypoxia is unknown. To examine the role of Hsp70 and Hsp32 in adaptation to chronic hypoxia we identified and characterized message levels and subcellular distribution of Hsp70i and Hsc70 protein and Hsp32 protein in hearts from human infants with cyanotic ( $\text{SaO}_2 < 85\%$ ) or acyanotic ( $\text{SaO}_2 > 95\%$ ) heart defects and in hearts from infant rabbits raised from birth in a hypoxic ( $\text{SaO}_2 < 85\%$ ) or normoxic ( $\text{SaO}_2 > 95\%$ ) environment. We then determined whether PKC, p38 MAPK, and JNK influence Hsp70 message expression and subcellular redistribution of Hsp70 protein in the signal transduction pathway activated by chronic hypoxia. Our studies indicate that in both infant human and rabbit hearts adaptation to chronic hypoxia increases expression of message for Hsp70i but not Hsc70. Increased message for Hsp70i translates to altered subcellular distribution of Hsp70i protein, with expression and subcellular localization of Hsp70i influenced by protein kinases. Hsp32 does not appear to be involved in adaptation of infant human and rabbit hearts to chronic hypoxia.

#### EXPERIMENTAL PROCEDURES

**Reagents.** Chelerythrine and SB203580 were purchased from Calbiochem. Curcumin was purchased from Sigma. The antibodies against inducible or constitutive Hsp70 were obtained from Calbiochem-Novabiochem. Antibodies against Hsp32 were obtained from Santa Cruz Biotechnology.

**Humans.** The use of human tissue in this study was approved by the Human Research and Review Committee at Children's Hospital of

going elective open heart surgery for congenital heart defects were prospectively recruited for this study. To determine whether Hsp70 and Hsp32 are activated by chronic hypoxia, the patients were divided into cyanotic and acyanotic groups according to blood oxygen saturation (cyanotic,  $\text{SaO}_2 < 85\%$ ; acyanotic,  $\text{SaO}_2 > 95\%$ ). All cyanotic patients were stable, with  $\text{SaO}_2 < 85\%$  for 24 h before surgery. There were no emergency operations performed on acutely hypoxic patients. Right atrial tissue (~200 mg) from infants was harvested at the time of surgical repair. The tissue was immediately frozen in liquid nitrogen and processed for Western analysis as described previously (12). Pre-operative characteristics are summarized in the Table.

**Rabbits.** Animals used in this study received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" formulated by the National Research Council, 1996. Infant rabbits were maintained from birth to 10 days of age in a hypoxic ( $\text{SaO}_2 < 85\%$ ) or normoxic ( $\text{SaO}_2 > 95\%$ ) environment as described previously (13) and then maintained from 10–30 days of age in a normoxic ( $\text{SaO}_2 > 95\%$ ) environment.

**Resistance to Ischemia.**—Resistance to myocardial ischemia was determined using an isolated perfused heart model as previously described (1). Hearts ( $n = 8$ /group) were perfused with bicarbonate buffer and left ventricular function continuously recorded as previously described (1). Hearts were then subjected to 30-min global ischemia followed by 35 min of reperfusion. Recovery of left ventricular developed pressure at 35 min of reperfusion was used to assess resistance to ischemia.

**Hsp70 mRNA and Hsp32 mRNA Extraction and Semi-quantitative RT-PCR.**—Constitutive and inducible Hsp70 gene expression was assessed in normoxic and chronically hypoxic hearts in infant human and rabbit with or without pharmacologic inhibition of protein kinases. Hsp32 gene expression was assessed in normoxic and chronically hypoxic hearts in infant human and rabbit. Total RNA from hearts was extracted using Trizol reagent (Invitrogen, Grand Island, NY) and quantified by spectrophotometry. 1 µg of total RNA was reverse-transcribed using SuperScript II RT (Invitrogen) in a total reaction volume of 20 µl. 1 µl of reverse transcription product (cDNA) was amplified using AmpliTaqDNA polymerase (PerkinElmer Life Sciences, Norwalk, CT) and 0.5 µl each of 10 µM inducible Hsp70 forward and reverse primers. The PCR cycle consisted of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min for a total of 35 cycles followed by 72 °C for 5 min. The primer sequences for Hsp70 and  $\beta$ -actin were: Rabbit Hsp70, forward 5'-3' CTCACGATCGACAGGAAGC, reverse 5'-3' AGCGTGTGCGGGGTCAGG (14); rabbit  $\beta$ -actin, forward 5'-3' GAAATCTGCGTCGATTAAG, reverse 5'-3' CTAGAAGCATTTGCGGGTGAACGATGGAGGGCC (14); human Hsc70 forward 5'-3' CCATGTGGTGAACCAAGATGAAG, reverse 5'-3' TCCATGCTCTCAGGATGGAAC (15); human Hsp70i forward 5'-3' CCATGTGGTGAACCAAGATGAAG, reverse 5'-3' CACCAAGCGTCAATGGAGAGAAC (15); and human  $\beta$ -actin, forward 5'-3' CCAGAGCAAGAGAGGATCC, reverse 5'-3' CTGTGGTGTGGTGAAGCTGTAG. The primer sequences for Hsp32 (heme oxygenase-1) were: forward 5'-3' CAGGACAGAGATCTGAGTTC and reverse 5'-3' GCTTCACATAGCGCTGCA (16). PCR products were visualized on 1% agarose gels stained with ethidium bromide. Control reactions were run using  $\beta$ -actin primers for 25 cycles as described above.

**SDS-PAGE and Western Blot Analysis.**—Expression of Hsp70 protein and Hsp32 protein was determined by Western analysis as described previously (1). Equal concentrations of protein were analyzed by SDS-PAGE and Western blotting using either specific antibodies against inducible or constitutive Hsp70 and Hsp32. The blots were developed by ECL<sup>TM</sup>. Densitometry was performed on each sample and analyzed using National Institutes of Health Image software. For studies on the control of Hsp70i protein distribution by protein kinases, the densitometric data was normalized so that the sum of the individual cytosolic and particulate fractions always equaled 100%, with subcellular distribution displayed in bar graph format.

**Immunohistochemistry Studies.**—Immunostaining was performed using a monoclonal (IgG<sub>1</sub>) antibody against Hsp70 (Stressgen Biotechnologies, clone C92P3A-5) at a concentration of 10 µg/ml. Purified mouse IgG<sub>1</sub> (Sigma) served as the negative control. Sections were processed using the Vectastain Elite ABC standard kit (Vector Laboratories) with biotinylated horse anti-mouse IgG (1:100, Vector) as secondary antibody. 2,3'-diaminobenzidine tetrahydrochloride (Vector) served as peroxidase substrate. Frozen hearts from normoxic and chronically hypoxic rabbits ( $n = 3$ /group) were cryosectioned to a thickness of 6 µm. Tissue sections were air-dried, fixed in 100% acetone for 10 min at -20 °C and rehydrated in Tris-buffered saline (control-containing

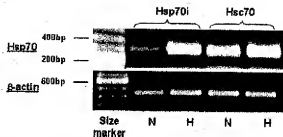


Fig. 1. mRNA levels for *Hsp70i* and *Hsc70* in normoxic and chronically hypoxic infant human heart. RT-PCR using primers for inducible *Hsp70* demonstrates that mRNA for *Hsp70i* was robustly expressed in chronically hypoxic hearts and minimally expressed in normoxic hearts. There were no changes in the level of *Hsc70* in chronically hypoxic and normoxic hearts. *β-actin* served as an internal control. Data shown are representative of four hearts analyzed for each condition studied. N = normoxic; H = chronically hypoxic.

blocked in 2.5% normal horse serum in TBSS for 1 h at room temperature before incubating overnight at 4 °C with anti-*Hsp70i* or negative control mouse IgG, at the corresponding dilution. Sections were washed 3 × 5 min in fresh changes of TBSS before application of the secondary antibody for 1 h at room temperature. Both primary and secondary antibodies were diluted in blocking buffer. Following application of the secondary antibody, saponin was omitted from all buffers and solutions. Sections were washed for 5 min in TBSS, then endogenous peroxidase was quenched using 1.5% hydrogen peroxide in TBSS for 5 min at room temperature. Following 3 × 5 min washes in TBSS, the Vectastain ABC Elite reagent was applied according to the kit protocol. Sections were again washed 3 × 5 min in fresh changes of TBSS. 3,3'-Diaminobenzidine tetrahydrochloride was applied to the sections per kit protocol and allowed to develop until optimal staining occurred. Finally, sections were washed in distilled water and counterstained with hematoxylin. Stained tissues were examined using brightfield microscopy with a Nikon E600 microscope and imaged using a Spot camera and software (Diagnostic Images, Inc.).

**Effect of PKC and MAPK Inhibitors—**Hearts from normoxic or chronically hypoxic rabbits were perfused in the Langendorff mode (13). Hearts ( $n = 10$ /group) were then perfused for 15 min with vehicle, chelerythrine (1  $\mu$ M/liter), SB203580 (15  $\mu$ M/liter), or curcumin (10  $\mu$ M/liter) as shown in Fig. 7. The free wall of the left ventricle was then processed to obtain cytosolic and particulate fractions (17) for Western analysis, as described previously (12).

**Statistical Analysis—**Statistical analysis was performed by use of repeated-measures analysis of variance with the Greenhouse-Geisser adjustment used to correct for the inflated risk of a type I error (13). If significant, the Mann-Whitney test was used as a second step to identify which groups were significantly different. After analysis of variance, the data were analyzed for differences related to multiple comparisons (13). Significance was set at  $p < 0.05$ .

#### RESULTS

**Hsp70i and Hsc70 mRNA Expression in Human Hypoxic Heart—**Because increased expression of mRNA and protein for *Hsp70* confers protection against cardiac ischemic injury (6, 18) and adaptation to chronic hypoxia also confers cardioprotection (1, 13), we determined the role of *Hsp70* in adaptation of the infant human heart to chronic hypoxia. Using semi-quantitative RT-PCR and their respective primers, inducible and constitutive levels of *Hsp70* mRNA were examined in normoxic and hypoxic infant human hearts. The levels of *Hsp70* mRNA between chronically hypoxic and normoxic hearts were compared by densitometric analysis after normalization to *β-actin*, the internal control. *Hsp70i* mRNA expression was robustly increased 4- to 5-fold in chronically hypoxic hearts but minimally expressed in normoxic hearts (Fig. 1). *Hsc70* mRNA was expressed in both chronically hypoxic and normoxic hearts. However, no detectable differences in the level of *Hsc70* mRNA expression were detected between chronically hypoxic and normoxic hearts (Fig. 1, upper panel). *β-actin* mRNA expression showed comparable density of bands for both normoxic and

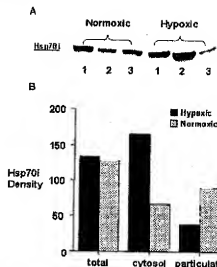


Fig. 2. Inducible *Hsp70* protein levels in normoxic and chronically hypoxic infant human heart. Analysis of total lysate, cytosolic, and particulate fractions was carried out as described under "Experimental Procedures." A, Western blot demonstrates that in hypoxic heart 80% of inducible *Hsp70* is localized in the cytosolic fraction with 20% localized in the particulate fraction. In normoxic hearts inducible *Hsp70* protein was almost equally distributed between the cytosolic and particulate fractions. 1 = total lysate; 2 = cytosolic fraction; and 3 = particulate fraction. B, densitometric measurement of inducible *Hsp70* protein in normoxic and hypoxic hearts representative of four experiments.

sizes for *Hsp70i*, *Hsc70*, and *β-actin* were 284, 283, and 436 bp, respectively. These data indicate infant human hearts adapt to the stress of chronic hypoxia by increasing expression of mRNA for *Hsp70i* but not *Hsc70*.

**Hsp70i and Hsc70 Protein Expression in Human Hypoxic Heart—**Because chronic hypoxia changes mRNA levels for *Hsp70*, we determined if this was translated into similar changes for protein levels for *Hsp70*. Total cell lysate, cytosolic, and particulate fractions from normoxic and hypoxic hearts were prepared. Protein content for constitutive and inducible *Hsp70* was determined by SDS-PAGE and Western blot analysis using monoclonal antibodies specific for inducible and constitutive *Hsp70*. We did not detect changes in *Hsp70i* and *Hsc70* protein levels in the total lysates obtained from chronically hypoxic and normoxic hearts (Figs. 2 and 3). However analysis of the cytosolic and particulate fractions indicates that, in chronically hypoxic human hearts, 80% of *Hsp70i* protein was localized in the cytosolic fraction with 20% of protein being in the particulate fraction. In normoxic human hearts, *Hsp70i* protein was distributed almost equally between the cytosolic and particulate fractions (40% versus 60%) (Fig. 2). In contrast, constitutive *Hsc70* protein was equally distributed between the cytosolic and particulate fractions of chronically hypoxic and normoxic infant human hearts, with no detectable differences in the total lysates (Fig. 3).

We examined whether expression of mRNA for *Hsp70* and subcellular distribution of *Hsp70* protein was related to the variability in clinical presentation of the two groups of patients studied (Table I). In all of the human infant hearts adapted to chronic hypoxia there was increased expression of mRNA for *Hsp70i* and redistribution of *Hsp70i* protein from the particulate to the cytosolic fraction. Increased message and subcellular redistribution of *Hsp70* protein did not occur in any of the normoxic hearts. Thus in all cases, the changes observed in *Hsp70i* translocation appear to be due to oxygen deprivation and not due to the underlying clinical presentation responsible

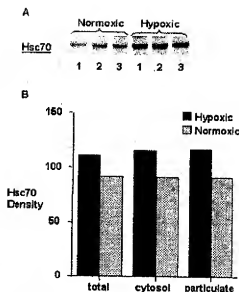


Fig. 3. Constitutive Hsp70 protein levels in normoxic and chronically hypoxic infant human heart. Analysis of total lysate, cytosolic, and particulate fractions was carried out as described under "Experimental Procedures." A, Western blot demonstrates constitutive Hsp70 distributed equally in cytosolic and particulate fractions in chronically hypoxic and normoxic hearts. 1 = total lysate; 2 = cytosolic fraction; and 3 = particulate fraction. B, densitometric measurement of constitutive Hsp70 protein in normoxic and chronically hypoxic hearts representative of four experiments.

TABLE 1  
Patient characteristics

	Cyanotic (n = 7)	Acyanotic (n = 8)
Age (months)		
Mean	4.8 ± 0.9	5.8 ± 1.2
Range	1 week to 9 months	1 month to 10 months
Body weight (kg)	3.9 ± 0.4	6.2 ± 0.5 <sup>a</sup>
Sex (male/female)	4/3	4/4
Pathology <sup>a</sup>		
HLHS	4	0
PAVC	0	3
DORV	1	0
AS	0	1
TOF	2	0
VSD	0	2
CAVC	0	2
Hemoglobin (g/dl)	15.6 ± 0.6	12.5 ± 1.3 <sup>a</sup>
Blood O <sub>2</sub> saturation (%)	73 ± 5	98 ± 1 <sup>a</sup>

<sup>a</sup> CAVC, complete atrio-ventricular canal; VSD, ventricular septal defect; TOF, tetralogy of Fallot; AS, aortic stenosis; DORV, double outlet right ventricle with transposition of the great arteries; PAVC, partial atrio-ventricular canal; HLHS, hypoplastic left heart syndrome.

<sup>b</sup>  $p < 0.05$  cyanotic versus acyanotic.

**Hsp70i and Hsc70 mRNA Expression in Rabbit Hypoxic Heart**—Using semi-quantitative RT-PCR and rabbit primers, the level of Hsp70 mRNA was determined in normoxic and hypoxic rabbit hearts. Transcripts for Hsp70i were elevated 4- to 5-fold in chronically hypoxic hearts compared with normoxic hearts (Fig. 4). Hsc70 mRNA was robustly expressed in both chronically hypoxic and normoxic rabbit hearts. However, adaptation to chronic hypoxia had no effect on expression of Hsc70 message levels compared with normoxic hearts (Fig. 4). Thus the adaptive response to chronic hypoxia in rabbit for Hsp70 mRNA was remarkably similar to that observed in human.

**Hsp70i and Hsc70 Protein Expression in Rabbit Hypoxic Heart**—There was no change in Hsp70i protein levels in the total lysates obtained from chronically hypoxic and normoxic

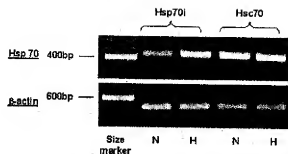


Fig. 4. mRNA levels for Hsp70i and Hsc70 in normoxic and chronically hypoxic infant rabbit heart. RT-PCR demonstrates inducible mRNA for Hsp70i robustly expressed in chronically hypoxic hearts and minimally expressed in normoxic hearts. mRNA levels for Hsc70 were unchanged in chronically hypoxic hearts compared with normoxic hearts.  $\beta$ -Actin served as an internal control. Data shown are representative of four hearts analyzed for each condition studied. N = normoxic; H = chronically hypoxic.

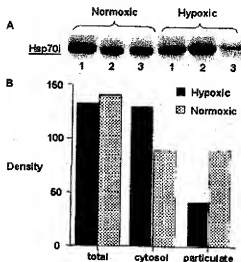
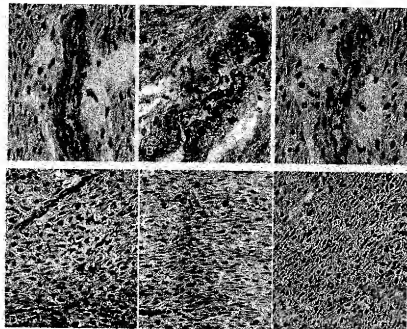


Fig. 5. Inducible Hsp70i protein level in normoxic and chronically hypoxic infant rabbit heart. Analysis of total lysate, cytosolic, and particulate fractions was carried out as described under "Experimental Procedures." A, Western blot demonstrates that in hypoxic heart 74% of Hsp70i protein is localized primarily in the cytosolic fraction with 26% localized in the particulate fraction. In normoxic hearts Hsp70i protein was equally distributed between the cytosolic fraction and particulate fractions. 1 = total lysate; 2 = cytosolic fraction; and 3 = particulate fraction. B, densitometric measurement of Hsp70i protein in normoxic and hypoxic hearts representative of four experiments.

Hsp70i protein was mainly redistributed to the cytosolic fraction, whereas in normoxic rabbit hearts, Hsp70i was equally distributed between the cytosolic and particulate fractions (Fig. 5). In chronically hypoxic and normoxic rabbit hearts, Hsc70 was equally distributed between the cytosolic and particulate fractions, with no detectable increase in Hsc70 protein levels in the total lysates. Our data show that Hsp70 signaling mechanisms activated by chronic hypoxia in infant rabbit hearts appear identical to those activated by cyanotic heart defects in infant human hearts. Moreover, this pattern of activation is also present in freshly excised rabbit hearts not subjected to perfusion prior to analysis. To complement the cellular studies, we performed immunohistochemistry to demonstrate the presence and redistribution of Hsp70i protein in normoxic and hypoxic infant rabbit hearts (Fig. 6). In normoxic and chronically hypoxic hearts we found that Hsp70i protein is abundantly present around the coronary vessels with pronounced and contiguous staining of the internal elastic lamina and

Fig. 6. Immunohistochemical staining of Hsp70i in hearts from normoxic and chronically hypoxic rabbits. Immunopositive staining is seen in coronary vessels and underlying vascular smooth muscle cells (A and B) and in cardiac myocytes (D and E) of both normoxic (A and D) and hypoxic (B and E) rabbits. Hsp70i immunolocalization in the cardiac myocytes in both normoxic (D) and hypoxic (E) rabbits are indicated by arrows. No positive immunostaining is seen in negative (mouse IgG<sub>1</sub>) controls (C and F). Magnification,  $\times 600$ .



our cellular studies we found that in normoxic hearts staining of Hsp70i protein was stronger in the membrane region and continued through the cytoplasm of the myocytes. Immunostaining of Hsp70i in chronically hypoxic hearts was less condensed in the membrane regions. There were no changes in overall intensity of staining in normoxic and chronically hypoxic hearts. Negative control sections incubated with mouse IgG<sub>1</sub> showed no positive immunostaining.

**Relationship between Hsp70 and Protein Kinases**—The mechanisms by which Hsp70 expression are controlled in infant hearts adapted to chronic hypoxia are unknown. We recently demonstrated that infant human and rabbit hearts adapt to chronic hypoxia through activation of PKC, p38 MAPK, and JNK signal transduction pathways (1). To determine the relationship of Hsp70 to these protein kinases in the signal transduction pathway activated by chronic hypoxia, infant rabbit hearts were perfused with inhibitors of PKC, p38 MAPK, and JNK (Fig. 7). Hearts were then examined for inducible and constitutive Hsp70 message levels by RT-PCR. Total cell lysate, cytosolic, and particulate fractions were analyzed for distribution of constitutive and inducible Hsp70 protein by Western blot analysis.

We confirmed adaptation to chronic hypoxia resulted in increased message levels for Hsp70i (Fig. 8). Perfusion of normoxic rabbit hearts with SB203580 (15  $\mu$ M), an inhibitor of p38 MAPK, had no effect on the message level for Hsp70i. However, SB203580 decreased Hsp70i mRNA levels in chronically hypoxic hearts. The PKC inhibitor chelerythrine (1  $\mu$ M) had no effect on Hsp70i message levels in normoxic hearts but reduced Hsp70i mRNA levels in chronically hypoxic hearts (Fig. 8). SB203580 and chelerythrine had no effect on constitutive Hsp70 mRNA levels in either normoxic or chronically hypoxic hearts (Fig. 9).

We then confirmed that adaptation to chronic hypoxia results in a shift in subcellular redistribution for Hsp70i protein from the particulate to the cytosolic fraction without any changes in the protein level for total cell lysates (Fig. 10, upper panel). To assist in between-group comparisons of data for subcellular Hsp70i protein distribution, we normalized the densitometric values for the cytosolic and particulate fractions and reported relative distribution in a bar graph (Fig. 10, lower

partly equal distribution of Hsp70i protein between the cytosolic and particulate fractions, with this distribution unaffected by perfusion of the heart with either SB203580, chelerythrine, or curcumin. In contrast, adaptation to chronic hypoxia resulted in the redistribution of Hsp70i protein from the particulate to the cytosolic fractions with this redistribution reversed by SB203580, chelerythrine, and curcumin (Fig. 10, lower panel). These data suggest that PKC, p38 MAPK, and JNK influence the cellular localization of Hsp70i in chronically hypoxic hearts.

**Hsp32 mRNA and Protein Expression in Human and Rabbit Hypoxic Heart**—Hsp32 (inducible, heme oxygenase-1 and constitutive, heme oxygenase-2) also belongs to the heat shock protein family. Hsp32 converts heme into bilirubin iron and carbon monoxide and is known to have cell-protective and anti-apoptotic properties (19, 20). We therefore reasoned that chronic hypoxia may activate Hsp32 and, using semi-quantitative RT-PCR and the respective primers for heme oxygenase-1, examined heme oxygenase-1 mRNA levels in normoxic and chronically hypoxic hearts from infant human and rabbit. Our results show that heme oxygenase-1 mRNA was expressed in both normoxic and chronically hypoxic hearts, with no detectable differences in the levels of heme oxygenase-1 mRNA expression between normoxic and hypoxic hearts in either infant human or infant rabbit (Fig. 11). We then examined heme oxygenase-1 and heme oxygenase-2 protein levels in normoxic and chronically hypoxic human and rabbit hearts. Protein content of heme oxygenase-1 and heme oxygenase-2 were determined by SDS-PAGE and Western blot analysis using monoclonal antibody specific for the two proteins. We did not detect changes in protein levels for either heme oxygenase between normoxic and chronically hypoxic hearts in both infant human and rabbit (Fig. 12). Thus Hsp32 does not appear to play a role in adaptation to chronic hypoxia in infant human and rabbit hearts.

**Association of Hsp70 with Resistance to Ischemia**—Hypoxia from birth increases the resistance of the infant rabbit heart to ischemia (1). However, the impact of subsequent exposure to normoxia during postnatal development upon cardioprotection is unknown. We determined whether the cardioprotective effects of chronic hypoxia from birth persist following subsequent

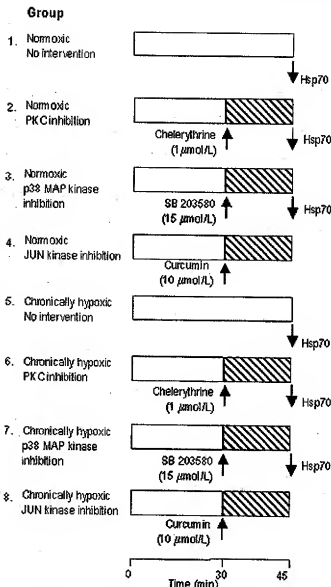


FIG. 7. Experimental protocol used to study the role of protein kinases in influencing *Hsp70* mRNA expression and *Hsp70* protein subcellular distribution in normoxic and chronically hypoxic infant rabbit hearts ( $n = 10/\text{group}$ ). Open boxes represent aerobic perfusion. Hatched boxes represent perfusion with protein kinase inhibitors.

of *Hsp70*i. At 10 days of age recovery of left ventricular developed pressure was higher in chronically hypoxic hearts ( $62 \pm 4\%$ ) than normoxic controls ( $46 \pm 4\%$ ). *Hsp70*i mRNA expression was elevated in hypoxic but not normoxic hearts (Fig. 1), and *Hsp70*i protein redistributed from the particulate to the cytosolic fraction (Fig. 2). At 30 days of age resistance to myocardial ischemia declined in normoxic hearts ( $34 \pm 3\%$ ) and was associated with equal distribution of *Hsp70*i between the cytosolic and particulate fractions (see Fig. 14). However, in hearts subjected to chronic hypoxia from birth to 10 days of age and then exposed to normoxia until 30 days of age, resistance to myocardial ischemia persisted ( $59 \pm 9\%$ ) with maintained elevation of mRNA for *Hsp70*i (Fig. 13) and maintained *Hsp70*i protein redistribution to the cytosolic fraction (Fig. 14). These studies indicate that cardioprotection conferred by adaptation to hypoxia from birth persists upon exposure to subsequent normoxia and is associated with cellular redistribution of

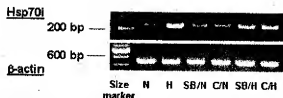


FIG. 8. Effect of pharmacologic inhibition of protein kinases on message levels for inducible *Hsp70* in normoxic and chronically hypoxic infant rabbit heart. RT-PCR using primers for *Hsp70*i demonstrates message levels 4- to 5-fold higher in untreated chronically hypoxic hearts compared with untreated normoxic hearts.  $\beta$ -Actin served as an internal control. Message levels for *Hsp70*i were reduced in chronically hypoxic but not normoxic hearts following 15-min perfusion with SB203580 and chelerythrine. Data shown are representative of four hearts analyzed for each condition studied. N = normoxic, untreated; H = hypoxic, untreated; SB/N = normoxic heart treated with SB203580; C/N = normoxic heart treated with chelerythrine; SB/H = hypoxic heart treated with SB203580; C/H = hypoxic heart treated with chelerythrine.



FIG. 9. Effect of pharmacologic inhibition of protein kinases on message levels for constitutive *Hsp70* mRNA in rabbit heart. RT-PCR using primers for *Hsc70* demonstrate equal message levels for both normoxic and chronically hypoxic hearts.  $\beta$ -Actin served as an internal control. No changes present in *Hsc70* message levels following SB203580 and chelerythrine treatment of normoxic and chronically hypoxic hearts. Data shown are representative of four hearts analyzed for each condition studied. N = normoxic, untreated; H = hypoxic, untreated; SB/N = normoxic heart treated with SB203580; C/N = normoxic heart treated with chelerythrine; SB/H = hypoxic heart treated with SB203580; C/H = hypoxic heart treated with chelerythrine.

#### DISCUSSION

Our study demonstrates that infant human hearts adapt to the stress of chronic hypoxia by increasing expression of *Hsp70*i message and redistributing *Hsp70*i protein from the particulate to the cytosolic fraction. In contrast, chronic hypoxia does not alter expression of *Hsc70* message or subcellular distribution of *Hsc70* protein. Infant rabbit hearts also adapt to chronic hypoxia by increasing expression of *Hsp70*i message and subcellular redistribution of *Hsp70*i protein. Thus the remarkably similar ways in which infant human and rabbit hearts adapt to chronic hypoxia suggest the rabbit may be useful to test adaptive mechanisms thought to occur in humans. Taken together, increased expression of *Hsp70*i message and subcellular redistribution of *Hsp70*i protein are important adaptive responses to chronic hypoxia. Because increased gene expression is often controlled by the sequential activation of cytoplasmic protein kinases, we sought to investigate whether activation of PKC and MAPK in hearts adapted to chronic hypoxia is related to *Hsp70*. In the present studies we have shown that *Hsp70*i mRNA expression and subcellular distribution of *Hsp70*i protein in chronically hypoxic infant rabbit hearts are influenced by several protein kinases, including PKC, p38 MAPK, and JNK.

Endogenous cellular protection against stresses such as prolonged periods of hypoxia may be conferred by several intracellular components such as protein kinases and heat stress proteins. These components are essential parts of the defense system within the heart. When the heart senses stress, ele-

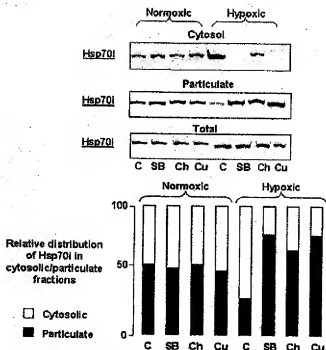


FIG. 10. Effect of pharmacologic inhibitors of protein kinases on inducible Hsp70 protein in rabbit heart total lysate, cytosolic, and particulate fractions. Western blot shows that, in hearts adapted to chronic hypoxia, Hsp70i translocates from the particulate to the cytosolic fraction. Pharmacologic inhibition of protein kinases had no effect on translocation of Hsp70i in normoxic hearts; however, in chronically hypoxic hearts SB203580, chelerythrine, and curcumin reversed the subcellular redistribution of Hsp70i protein caused by chronic hypoxia. C = untreated control; SB = SB203580-treated; Ch = chelerythrine-treated; Cu = curcumin-treated.

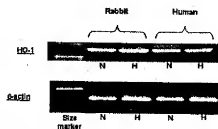


FIG. 11. mRNA levels for Hsp32 (Hsp70) in normoxic and chronically hypoxic rabbit and human heart. RT-PCR using primers for Hsp32 demonstrates that there were no changes in the level of Hsp70 mRNA in normoxic and chronically hypoxic heart.  $\beta$ -Actin served as an internal control. Data shown are representative of four hearts analyzed for each condition studied. N = normoxic; H = chronically hypoxic.

that myocardial cells adapt to the stress of chronic hypoxia by activation of multiple protein kinases (1). Chronically hypoxic human infant and infant rabbit hearts activate PKC $\epsilon$ , which is manifest by the translocation of the PKC $\epsilon$  isoform from the cytosolic to the particulate fraction. PKC $\epsilon$  but not the  $\alpha$ ,  $\beta$ ,  $\delta$ ,  $\gamma$ , and  $\zeta$  isoforms of PKC were phosphorylated and translocated in hearts adapted to chronic hypoxia (1). Chronic hypoxia also results in activation of p38 MAPK and JNK but not p42/p44 MAPK in both human and rabbit hearts. We also showed activation of p38 MAPK by chronic hypoxia activates MAPKAP-2, which in turn activates Hsp27. Furthermore, chronic hypoxia caused phosphorylation of activating transcription factor ATF-2, a substrate for p38 MAPK (1). The present study shows that activation of PKC, p38 MAPK, and JNK appears to influence expression of mRNA for Hsp70i and subcellular distribution of Hsp70i protein in hearts adapted to chronic hy-

poxy in cellular protection or repair of injury. Our findings that Hsp70i is regulated by protein kinases supports the work of Das *et al.* (21) who demonstrated that stress induced by repeated cycles of ischemia and reperfusion in the heart results in activation of the p38 MAPK-MAPKAP2 pathway and induction of Hsp70, which is blocked by both genistein and SKF86002. We did not detect any differences in either inducible or constitutive Hsp32 isoforms present in normoxic and chronically hypoxic hearts for both infant rabbit and human. These data suggest selective activation of some but not all small molecular weight Hsps in response to the stress of chronic hypoxia in infant human heart.

Perfusion of chronically hypoxic infant rabbit hearts with pharmacologic inhibitors of protein kinases for only 15-min results in reduced expression of Hsp70i message and a corresponding redistribution of Hsp70i protein from the cytosolic to the particulate fraction. Inhibition of protein kinases had no effect on expression of mRNA for Hsp70 or distribution of Hsp70 protein within the cell. The short time period required for inhibition of Hsp70i mRNA suggests a high turnover rate for Hsp70. SB203580, a selective p38 MAPK inhibitor, decreases Hsp70i message and redistributes Hsp70i protein in chronically hypoxic but not normoxic hearts. These findings suggest that p38 MAPK activity is essential for hypoxia-induced up-regulation of Hsp70, which may contribute to the subsequent cardioprotection. Our observation confirms previous findings in chronically hypoxic astrocytes where SB203580 attenuated the increase in Hsp70 message (22). Inhibition of p38 MAPK with SB203580 also abolishes the effect of Hsp70 on cytokine-induced accumulation of NOS2 message (23). Furthermore, hypertonic induction of Hsp70 message in the kidney is inhibited by SB203580 (24). These findings suggest p38 MAPK activation is essential for adaptation to stress by induction of heat shock proteins. However, inhibition of Hsp70 by SB203580 may be the result of inhibition of HSF-1 phosphorylation. The effect of staurosporine, a potent PKC inhibitor, on HSF-HSE binding indirectly by heat in HT-29 cells was examined by Erdos and Lee (25). They demonstrated that staurosporine treatment did not alter heat-induced HSF-HSE-binding ability and concluded that staurosporine did not inhibit HSF-1 phosphorylation. However other authors have demonstrated that staurosporine suppresses the accumulation of Hsp70 mRNA and Hsp70 in HT-29 cells induced by heat. They reasoned that Hsp70 mRNA suppression was the result of decrease in initiation and elongation activity of the Hsp70 gene. Similarly, general inhibitors of PKC, PKA, and PKG (e.g. H-7 and H-8) have been shown to suppress heat-induced accumulation of Hsp70 mRNA by Lee *et al.* (26) and have suggested that protein kinases contribute to the synthesis of Hsp70 mRNA via HSF-1 phosphorylation without showing which protein kinase was involved. It has been reported that regulation of HSF-1 phosphorylation via PKC and H-7 suppresses the DNA binding of HSF-1 (7). In addition to protein kinases, other genes have been shown to be important in regulating Hsp70 expression in response to stress. In an elegant study by Zhao *et al.* (27) on the role of the double-stranded RNA-dependent protein kinase gene (*pkr*), this gene was shown to be essential in the heat stress response and is involved in regulating expression of Hsp70 and other heat shock proteins through mRNA stabilization.

Amrani *et al.* (6) and Okubo *et al.* (18) have shown that introduction of exogenous mRNA for Hsp70 using gene transfer translates to an increase in Hsp70 protein content. However, these studies performed on total cell lysates did not determine whether increased Hsp70 protein expression results in subcellular redistribution of Hsp70 protein. In our study, adaptation

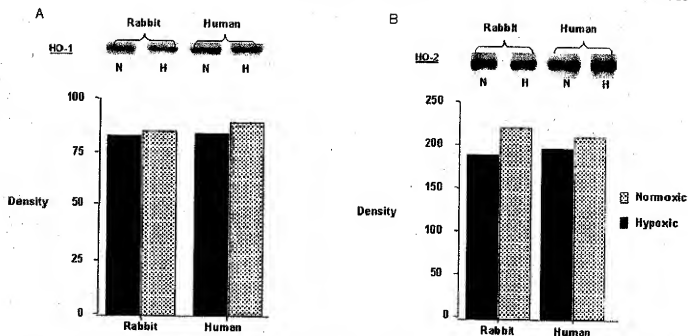


FIG. 12. Hsp32 (HO-1 and HO-2) protein levels in normoxic and chronically hypoxic rabbit and human heart. Preparation of hearts was carried out as described under "Experimental Procedures." A, Western blot demonstrates protein level and densitometric measurement of HO-1 from normoxic and chronically hypoxic rabbit and human hearts. B, Western blot demonstrates protein level and densitometric measurement of HO-2 from normoxic and chronically hypoxic rabbit and human hearts. Densitometric measurements of Hsp32 protein in normoxic and hypoxic hearts are representative of four experiments. N = normoxic; H = chronically hypoxic.

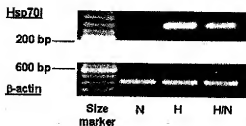


FIG. 13. mRNA levels for *Hsp70i* in hearts from rabbits exposed to chronic hypoxia and then normoxia. RT-PCR shows that mRNA for *Hsp70i* was highly expressed in hearts from infant rabbits maintained from birth to 10 days of age in a hypoxic environment and in rabbits subsequently maintained in a normoxic environment from 10 to 30 days of age but minimally expressed in hearts from infant rabbits maintained from birth to 30 days of age in a normoxic environment.  $\beta$ -Actin served as an internal control. Data shown are representative of four hearts analyzed for each condition studied. N = normoxic (30 days old); H = hypoxic (10 days old); HN = hypoxic from birth to 10 days of age and then maintained in a normoxic environment from 10 to 30 days of age.

mRNA for *Hsp70* without a corresponding increase in protein content in total cell lysates. However, we detected a substantial change in the extent of subcellular distribution for *Hsp70i* protein in chronically hypoxic hearts, with *Hsp70i* redistribution controlled by several protein kinases. We believe this is the first evidence of a protein kinase mediation of mRNA expression that translates to subcellular redistribution of *Hsp70* protein in response to the stress of chronic hypoxia in the infant human and rabbit heart. Di *et al.* (28) have shown that, in lymphocytes, *Hsp70* undergoes a similar dynamic and reversible change in subcellular distribution in response to heat stimuli. Furthermore, *Hsp70* protein expression and distribution were controlled by PKC, which resulted in aggregation of *Hsp70* with PKC, which was inhibitable by calphostin C. Our immunohistochemistry studies confirmed the presence and distribution of *Hsp70i* protein in normoxic and chronically hypoxic infant rabbit hearts. The results of these studies provide addi-

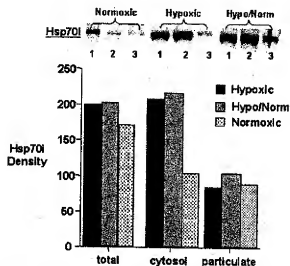


FIG. 14. Inducible *Hsp70* protein levels in hearts from rabbits exposed to chronic hypoxia and then normoxia. Analysis of total lysate, cytosolic, and particulate fractions was carried out as described under "Experimental Procedures." A, Western blot demonstrates that in hearts from rabbits maintained in a hypoxic environment from birth to 10 days of age *Hsp70i* protein is located primarily in the cytosolic fraction. More importantly, this distribution pattern for *Hsp70i* is maintained when the rabbit is subsequently maintained in a normoxic environment from 10 to 30 days of age (*Hypo/Norm* group). In hearts from rabbits maintained in normoxia from birth to 30 days of age, *Hsp70i* is equally distributed in the cytosolic and particulate fractions. 1 = total lysate; 2 = cytosolic fraction; 3 = particulate fraction. B, densitometric measurement of *Hsp70i* protein in each group is representative of four experiments.

normoxic and chronically hypoxic hearts, we found that *Hsp70i* protein is abundantly present around the coronary vessels. We also found that *Hsp70i* protein is abundantly present and re-distributed between normoxic and chronically hypoxic hearts with staining of chronic hearts for *Hsp70i* less condensed in the

immunohistochemistry to localize proteins within tissues. Staining of tissue is generally more difficult, because the epitope recognized by the antibody may not be well exposed due to the complex architecture of the cardiac fibers. To address this challenge we evaluated antibodies from several vendors to select a suitable probe for use in the immunohistochemistry studies.

The major objective of our study was to examine the role of Hsp70 and Hsp32 in adaptation of the infant rabbit and human heart to chronic hypoxemia. It was not our intention to directly relate Hsp70 to cardioprotection. However, to address this important question we performed additional studies on the association of Hsp70 with resistance to ischemia. Our data suggest that cardioprotection conferred by adaptation to hypoxia from birth persists upon exposure to subsequent normoxia and is associated with cellular redistribution of Hsp70i.

Myocardial ischemia and reperfusion injury is an important problem in the clinical setting. Surgical intervention, pharmacological therapy, and physical exercise have been prescribed for the treatment of patients with cardiovascular disease. To demonstrate the importance of Hsp70 in cardioprotection, Hsp70-transgenic mice have been generated (29). In these mice, the rat Hsp70 gene is placed under control of a human cytomegalovirus intermediate-early enhancer and  $\beta$ -actin promoter, resulting in strong constitutive expression of Hsp70 in cardiac muscle, skeletal muscle, and brain. These mice display resistance against several models of heart ischemic injury (29–31). Also by means of adenoviruses, the protecting capacity of Hsp70 against myocardial ischemia was demonstrated (32). Another argument for a cytoprotective role of Hsp70 was provided by the construction of HSF-1 knockout mice (33). HSFs regulate the stress-inducible synthesis of HSPs (34). HSF-1 knockout mice show an increased sensitivity to lipopolysaccharide-induced toxicity and lethality (35). Finally, there are numerous reports clearly demonstrating a cytoprotective role of Hsp70 *in vitro* by means of heat shock induction or Hsp70 overexpression (36–38) against toxicity induced by several cytokines. Recently, however, attention has focused on gene-based therapies with Hsp70 to confer cardioprotection in the setting of surgical ischemia (6, 8, 39). Thus exploitation of the Hsp70 signaling pathway may afford cardioprotection to human infants undergoing repair of congenital heart defects.

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